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**Acute lung injury: Apoptosis in effector and target cells of the upper and lower airway
compartment upon endotoxin and hypoxia-induced injury**

INAUGURAL-DISSERTATION

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1 Abstract

Background: Apoptosis has been considered as an underlying mechanism in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). Suggestions are now emerging to assume that similar cell types might experience different apoptosis degrees upon injury. Endotoxin-induced injury is an experimental *in vitro* and *in vivo* model closely resembling ALI and ARDS in humans. Additionally, it is known from previous studies that hypoxia can exert a pro-inflammatory effect on the lung.

Methods: Apoptosis was determined in effector cells (alveolar macrophages, neutrophils) and target cells (tracheobronchial and alveolar epithelial cells) of the respiratory compartment, measuring caspase-3 activity, which is a crucial mediator of apoptosis. Cells were stimulated with lipopolysaccharide (LPS) for 4 h or cells were exposed to 5% oxygen for 4 h. Camptothecin was used as a positive control for induction of apoptosis.

Results: Apoptosis rate of alveolar macrophages was increased by 75% ($p < 0.05$) in the presence of LPS, while hypoxia did not affect caspase-3 activity. Neutrophils, however, showed a decreased apoptosis rate of 39% under hypoxia ($p < 0.05$). LPS did not change caspase-3 activity in neutrophils. Tracheobronchial epithelial cells experienced an enhanced caspase-3 activity upon LPS stimulation (increase of 121%, $p < 0.05$) with no change under hypoxia. Similar results were found in alveolar epithelial cells with a LPS-induced increase of apoptosis rate of 45% ($p < 0.05$) and an unaffected caspase-3 activity under hypoxia.

Discussion: While alveolar macrophages, tracheobronchial and alveolar epithelial cells demonstrated a similar pattern regarding changes of apoptosis rate upon injury, neutrophils react differently. The biological impact of this observation is under evaluation. Further experiments have to be performed to determine the possible pro- or anti-inflammatory role of apoptosis of cells in the respiratory compartment upon injury.

2 Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) cause severe respiratory failure and death in critically ill patients. The development of ALI/ARDS is associated with several clinical disorders including direct pulmonary injury from pneumonia and aspiration as well as indirect pulmonary injury from trauma or sepsis. Additionally ventilatory support including mechanical stress on respiratory epithelial cells may also negatively affect lung tissue and contribute to the development of ALI/ARDS (1). Although knowledge about mechanisms leading to ALI/ARDS has increased, no specific and successful treatment options exist to date and thus the mortality rate remains high in patients with ALI/ARDS (2).

The airway compartment with alveolar macrophages and epithelial cells as the predominant cell types is a physiological barrier to a variety of environmental agents including gases, particulates and microbes. An injury to lung tissue involving the airway compartment can lead to serious illness and life-threatening conditions such as ALI/ARDS. Alveolar macrophages are located at the air-tissue interface in the lung and are therefore the first cells, which interact with inhaled organisms and antigens (3). A central factor in the pathogenesis of inflammatory lung disease is the locally elevated number of alveolar macrophages. It has been shown in several lung injury models that activated pulmonary macrophages release cytokines as well as the chemokines (4, 5). All these inflammatory mediators together play a crucial role in the orchestration of an inflammatory response, particularly in neutrophil recruitment. Neutrophil sequestration and migration into alveoli remain pathohistological hallmarks of ARDS, with neutrophils being key effector cells, which further destruction of lung tissue (6).

Lipopolysaccharide (LPS)-induced lung injury is a very useful experimental model for the investigation and characterization of those immunopathogenic changes and mechanisms in acute lung injury. LPS, a component of the outer membrane of gram negative bacteria, is capable of inducing severe lung injury. The response to LPS in the lung results in an acute inflammatory reaction regulated by the coordinated function of cytokines, chemokines and adhesion molecules.

The process of programmed cell death, or apoptosis is known to play a major regulatory role in maintaining many biologic processes, not least of which is the inflammatory response, such as in ALI/ARDS. Two major apoptosis pathways in mammalian cells are known so far: 1) the

intrinsic or mitochondrial pathway with involvement of Bcl-2 at the outer membrane of mitochondria and cytochrome c release 2) extrinsic or death receptor pathway with activation of caspase-8 upon binding of death activator to Fas- and TNF-receptor at the surface of the cell. Both pathways converge at the level of caspase-3 activation (7). Apoptosis results in destruction of proteins by caspases as well as in fragmentation of the DNA. Cell volume decreases and finally apoptotic cells are eliminated by phagocytes. Inappropriate activation or inhibition of apoptosis can lead to disease either because ‘undesired’ cells develop prolonged survival or because ‘desired’ cells die prematurely (8).

Purpose of this study was to evaluate endotoxin and hypoxia-induced apoptosis rate of effector cells (alveolar macrophages and neutrophils) as well as target cells (tracheobronchial epithelial cells and alveolar epithelial cells).

3 Material and Methods

Animals

Specific pathogen-free male Wistar rats (250-300 g) were purchased from Janvier (Le Genest-St. Isle, France). Rats were anesthetized with subcutaneously administered Hypnonorm® (fentanyl/fluonisone; Janssen, Beerse, Belgium) 0.25 ml/kg body weight, Domitor® (medetomidine; Pfizer, Inc., Westchester, PA, USA) 0.25 ml/kg body weight and 0.1% atropine 0.05 ml/kg body weight. All animal experiments and animal care were approved by the Swiss Veterinary Health Authorities.

Alveolar macrophages

Mac cells (CRL-2192, American Type Culture Collection, Rockville, MD) were established from normal Sprague-Dawley rat alveolar macrophage cells obtained by lung lavage, cloned and subcloned three times. The cells exhibit characteristics of macrophages and are sensitive to endotoxin.

Isolation of neutrophils

Human neutrophils (PMN) were isolated by gradient centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Dubendorf, Switzerland) followed by 1% dextran sedimentation for 1 h to separate neutrophils from erythrocytes, as described previously (9). After centrifugation of the supernatant, contaminating erythrocytes were lysed with distilled water followed by the addition of 2.7% NaCl to stop hypotonic lysis. Neutrophils were washed with phosphate-buffered saline (PBS) and resuspended at a total concentration of 2×10^6 PMN/ml in DMEM with 1% FBS.

Isolation and culture of TBEC

Trachea and bronchial parts of the respiratory system were excised, ligated at the distal ends, filled with 0.01% protease type XIV (Sigma, Buchs, Switzerland) and incubated overnight at 4°C (10). TBEC were flushed out with fetal bovine serum (FBS), washed twice, and incubated in airway epithelial cell basal medium (PromoCell) with 10% FBS in 96-well plates, previously coated with 50 µg/ml rat tail collagen (Sigma, Buchs, Switzerland) for 30 min at room temperature. Cells reached 100% confluency within 3 days. Purity was verified using periodic acid-Schiff staining (>98%). Epithelial cell character was also confirmed by a pathologist at the University Hospital of Zurich.

Alveolar epithelial cells (AEC)

L2 cells (CCL 149, American Type Culture Collection, Rockville, MD) are isolated cell lines derived through cloning of adult female rat lung of alveolar epithelial cell type II origin (11). The cells were cultured in Dulbecco's modified Eagle's Medium (DMEM; Invitrogen AG, Basel, Switzerland), supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% HEPES buffer and grown in uncoated 96-well plates (Corning Inc., Corning, NY) to more than 95% confluence. Prior to cell stimulation, the medium was changed to DMEM with 1% FBS.

Stimulation with LPS

Cells were washed twice with warm phosphate-buffered saline (PBS) and incubated with LPS from *Escherichia coli* serotype 055:B5 (LPS; 20 µg/mL; Sigma-Aldrich, Buchs, Switzerland) (or PBS as a control) for 4 h at 37°C.

Hypoxia

A cell incubator (Bioblock, Ittigen, Switzerland) adjustable to different oxygen concentrations by insufflation of nitrogen (N₂) was used as a hypoxic cell chamber. The concentrations were monitored continuously by an oxygen sensor. Experiments were performed with 5% oxygen and 5% CO₂ at 37°C. For control cells, an incubator (Bioblock) with 21% O₂, 5% CO₂ at 37°C was used. For our studies, L2 cells, Mac and isolated PMN were plated in 96-well tissue culture plates (Corning, New York, NY, USA) and exposed to 5% O₂ for 4 h.

Fluorometric assay for caspase-3 activity

For the caspase assays, TBEC were isolated and grown to confluence in 96-well plates, as well as L2 and Mac and stimulated with LPS (20 µg/ml) or camptothecin (4 µM) for 4 h. Caspase-3 activity was determined by measuring proteolytic cleavage of the fluorogenic caspase-3 substrate Ac-Asp-Glu-Val-Asp-AMC (Merck, Darmstadt, Germany). Cells were incubated for 1 h at 37°C with 125 µM substrate. The fluorescence of the cleaved reporter group was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm. Camptothecin (an extract of the Chinese tree *Camptotheca acuminata*) is a potent inhibitor of topoisomerase I, a molecule required for DNA synthesis. Camptothecin has been shown to induce apoptosis and was therefore used for positive controls.

Determination of lactate dehydrogenase

Viability of cells was determined by the trypan blue dye exclusion assay. In addition, results were confirmed with a cytotoxicity assay (determination of lactate dehydrogenase) (Promega, Madison, WI) as described previously (12).

Statistical analysis

Results are expressed as means \pm SEM. All experiments were conducted at least 3 times. ANOVA was performed to assess the statistical significance of differences. P values < 0.05 were considered significant.

4 Results

Hypoxia- and endotoxin-induced apoptosis in effector cells

To determine a possible effect of hypoxia on apoptosis in alveolar macrophages and neutrophils, caspase-3 as the key enzyme in the final pathway was determined. Interestingly, the two cell types, although belonging to the group of effector cells, did not experience the same changes. While under hypoxic conditions apoptosis rate did not change in alveolar macrophages compared to control cells, caspase-3 activity increased by 75% in the LPS group ($p < 0.05$) (**Fig. 1**). Neutrophils, however, showed less caspase-3 activity under hypoxia in comparison to control cells (39% decrease, $p < 0.05$). With LPS, neutrophils experienced no change in apoptosis rate (**Fig. 2**).

Hypoxia- and endotoxin-induced apoptosis in target cells

Hypoxia did not alter apoptosis rate in tracheobronchial epithelial cells. Stimulation with LPS increased caspase-3 activity by 121% ($p < 0.05$) (**Fig. 3**). Alveolar epithelial cells as possible target cells showed a similar apoptosis pattern as tracheobronchial epithelial cells and alveolar macrophages. Hypoxia did not induce changes in apoptosis rate in alveolar epithelial cells, while LPS increased caspase-3 activity (45%, $p < 0.05$) (**Fig. 4**).

Hypoxia- and endotoxin-induced cell death in effector cells

Cell death rate was determined to first exclude an interference with the apoptosis results and second to evaluate the direct effect of hypoxia and endotoxin, respectively, on cell death. Spontaneous cell death in alveolar macrophages after a 4 h incubation rate was 22% (**Fig. 5**). Similar results were found in these cells under hypoxia and LPS with no statistically significant differences compared to control cells. In neutrophils, basal death rate was 20% with no changes under different conditions (**Fig. 6**).

Hypoxia- and endotoxin-induced cell death in target cells

Tracheobronchial epithelial cells had a spontaneous death rate of 27% (**Fig. 7**). But also in these cells, death rate was not altered under hypoxia or LPS. Similar results were found for alveolar epithelial cells with no change in cell death rate upon injury (**Fig. 8**).

5 Discussion

Numerous studies have been conducted to better understand ALI/ARDS. Cell death has been demonstrated to play a key role in the lung during the pathogenesis of ALI/ARDS. In this study we focused on different cell types of the respiratory compartment and determined apoptosis and necrosis *in vitro* in the model of hypoxia- or endotoxin-induced injury. While alveolar macrophages, alveolar epithelial cells, and tracheobronchial epithelial cells showed the same apoptosis pattern with increased apoptosis rate under stimulation with LPS and no change upon hypoxic condition, an attenuated apoptosis rate was seen in neutrophils under hypoxia and no change after stimulation with endotoxin.

Programmed cell death is a process by which cells ‘commit suicide’ through apoptosis or other alternative pathways. Cell death occurs at a specific point in the developmental process and is therefore considered as ‘programmed’. It can also be triggered by external stimuli, such as soluble cell death ligands, which are released during inflammatory responses, or intrinsic stimuli, resulting from alteration of cellular function and metabolism. Apoptosis is characterized by cell shrinkage and formation of apoptotic bodies. Various biochemical features of apoptosis have been identified, which have been used frequently as an indication for apoptosis, such as caspase activation, DNA fragmentation, and externalization of phosphatidylserine, a cell surface marker for phagocytosis (7). Caspases are the most extensively studied proteases that are activated during apoptosis. They exist as inactive protease precursors within cells and can be activated by themselves or by other proteases. Both an intrinsic and extrinsic pathway end up with activation of caspase-3.

LPS has been commonly used as a tool to study the mechanisms of ALI in cultured cells and in animals. In a model of LPS administration in mice, extended apoptosis was observed in vascular endothelial cells, but also in epithelial cells (13). Our results support these findings with increased apoptosis rate in epithelial cells. Caspase activity of neutrophils, however, was not changed upon LPS stimulation at this early time point of injury. Interestingly, a recent study showed that neutrophil apoptosis seems to be inhibited in patients with ALI/ARDS (14). In a different model of ALI, induced by intravascular injection of oleic acid to simulate pulmonary fat embolism-induced ALI, at 1 and 4 h following oleic acid injection a massive neutrophil response was found in the lung, without any evidence of apoptosis. 24 h later, in the early resolution stage, intense neutrophil apoptosis was observed (15). Therefore, it might be

concluded that neutrophils experience a different apoptosis response than the other cells of the respiratory compartment at this early time point of injury. Alveolar macrophages, however, seem to have a similar reaction pattern as epithelial cells (16). As previously described, among the different bacterial cell wall components, LPS seems to be the only inducer of apoptosis in human alveolar macrophages.

Under hypoxic conditions apoptosis rate of epithelial cells and alveolar macrophages did not change. Again, neutrophils experience a different reaction regarding apoptosis rate compared to the other cell types. Hypoxia decreased caspase-3 activity in neutrophils. Current data indicate that many factors operating at the inflamed site as hypoxia and acidosis serve a dual function in both priming and activating neutrophils and delay apoptosis (17).

Hypoxic or endotoxin-induced injury did not induce cell death in all cell types. This observation might not be surprising as 4 hours of stimulation are rather short. Necrosis might be considered as the common destiny of cell death, therefore also for apoptosis. The routes of cell death could be interrelated based on underlying mechanisms and conditions (18). Taking this concept into account, one should pay more attention to the pathways such as apoptosis that eventually lead to necrosis. The concept may help to explain why blocking these earlier states of cell death may improve the organ function by preventing necrosis.

In conclusion, our data show, that the three cell types from the respiratory compartment alveolar and tracheobronchial epithelial as well as alveolar macrophages show the same pattern of apoptosis upon exposure to hypoxia and endotoxin. The apoptotic answer of neutrophils, however, is different. The functional implications of these inflammatory answers have further to be analyzed.

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7 Figure Legend

Figure 1

Determination of apoptosis rate in alveolar macrophages. Caspase-3 activity was measured in control cells, after exposure to 5% oxygen, or exposure to 20 µg/ml lipopolysaccharide (LPS) for 4 h. Camptothecin was used as a positive control for apoptosis. Values are mean +/- SEM from 5 experiments.

* $P < 0.05$ between control and hypoxia.

Figure 2

Determination of apoptosis rate in neutrophils. Caspase-3 activity was measured in control cells, after exposure to 5% oxygen, or exposure to 20 µg/ml lipopolysaccharide (LPS) for 4 h. Camptothecin was used as a positive control for apoptosis. Values are mean +/- SEM from 5 experiments.

* $P < 0.05$ between control and LPS.

Figure 3

Determination of apoptosis rate in tracheobronchial epithelial cells. Caspase-3 activity was measured in control cells, after exposure to 5% oxygen, or exposure to 20 µg/ml lipopolysaccharide (LPS) for 4 h. Camptothecin was used as a positive control for apoptosis. Values are mean +/- SEM from 5 experiments.

* $P < 0.05$ between control and LPS.

Figure 4

Determination of apoptosis rate in alveolar epithelial cells. Caspase-3 activity was measured in control cells, after exposure to 5% oxygen, or exposure to 20 µg/ml lipopolysaccharide (LPS) for 4 h. Camptothecin was used as a positive control for apoptosis. Values are mean +/- SEM from 5 experiments.

* $P < 0.05$ between control and LPS.

Figure 5

Cell death rate in alveolar macrophages. Lactate dehydrogenase (LDH) activity was measured in supernatants of control cells, after exposure to 5% oxygen, or exposure to 20 µg/ml lipopolysaccharide (LPS) for 4 h. Some wells were lysed to determine 100% release of LDH. LDH in supernatants was correlated to total LDH. Camptothecin was used as a positive control for apoptosis. Values are mean \pm SEM from 5 experiments.

Figure 6

Cell death rate in alveolar neutrophils. Lactate dehydrogenase (LDH) activity was measured in supernatants of control cells, after exposure to 5% oxygen, or exposure to 20 µg/ml lipopolysaccharide (LPS) for 4 h. Some wells were lysed to determine 100% release of LDH. LDH in supernatants was correlated to total LDH. Camptothecin was used as a positive control for apoptosis. Values are mean \pm SEM from 5 experiments.

Figure 7

Cell death rate in tracheobronchial epithelial cells. Lactate dehydrogenase (LDH) activity was measured in supernatants of control cells, after exposure to 5% oxygen, or exposure to 20 µg/ml lipopolysaccharide (LPS) for 4 h. Some wells were lysed to determine 100% release of LDH. LDH in supernatants was correlated to total LDH. Camptothecin was used as a positive control for apoptosis. Values are mean \pm SEM from 5 experiments.

Figure 8

Cell death rate in alveolar epithelial cells. Lactate dehydrogenase (LDH) activity was measured in supernatants of control cells, after exposure to 5% oxygen, or exposure to 20 µg/ml lipopolysaccharide (LPS) for 4 h. Some wells were lysed to determine 100% release of LDH. LDH in supernatants was correlated to total LDH. Camptothecin was used as a positive control for apoptosis. Values are mean \pm SEM from 5 experiments.

Figures

Figure 1

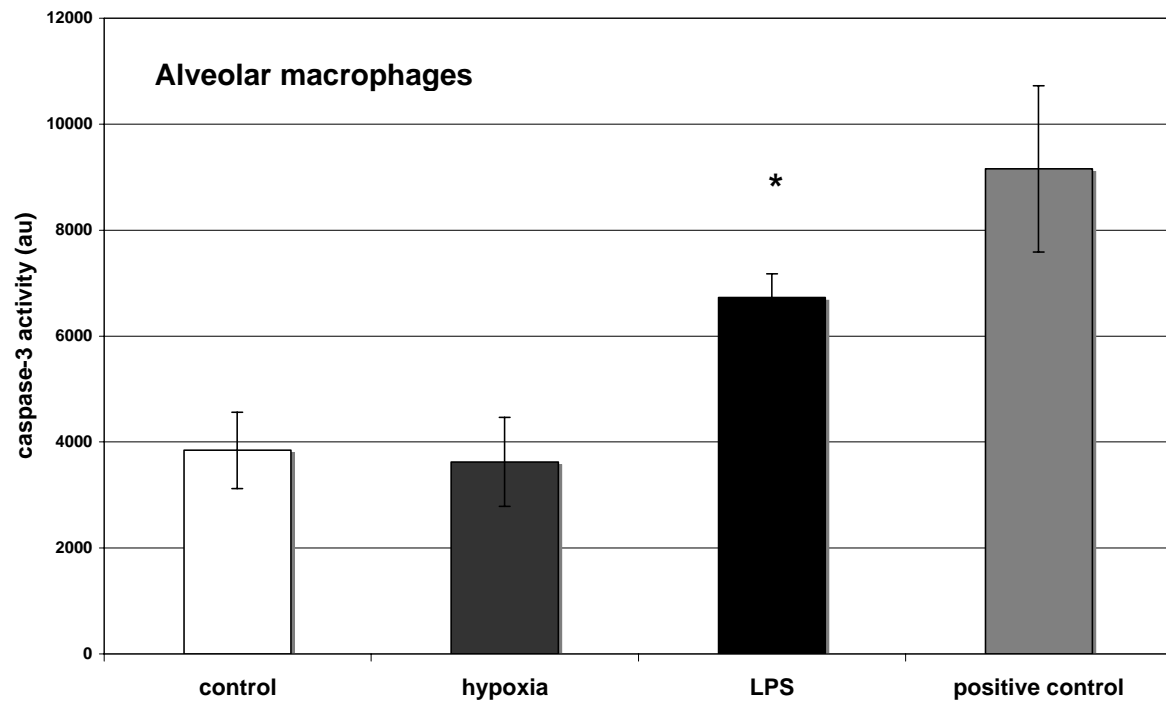


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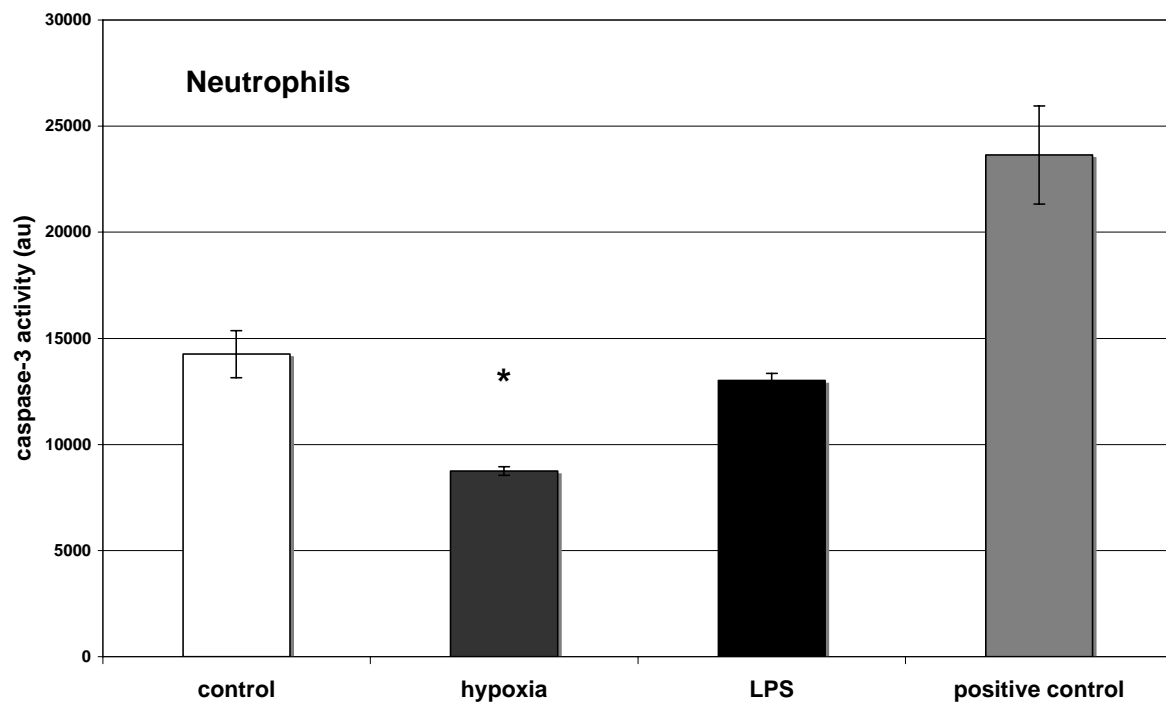


Figure 3

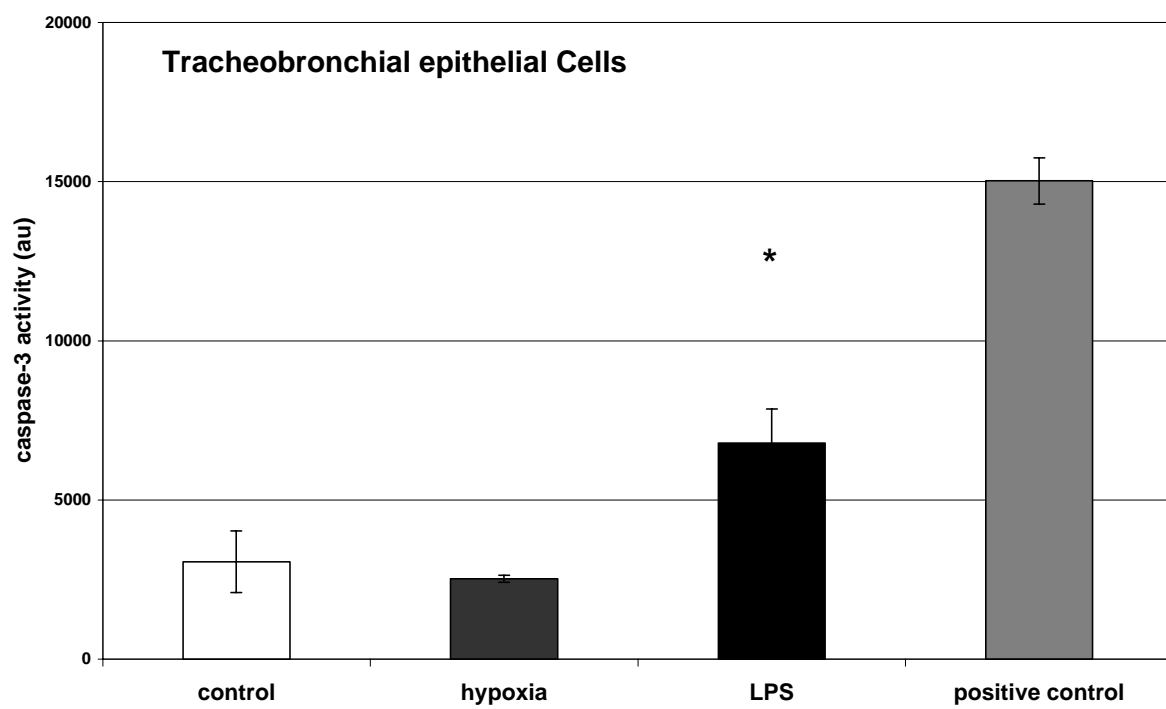


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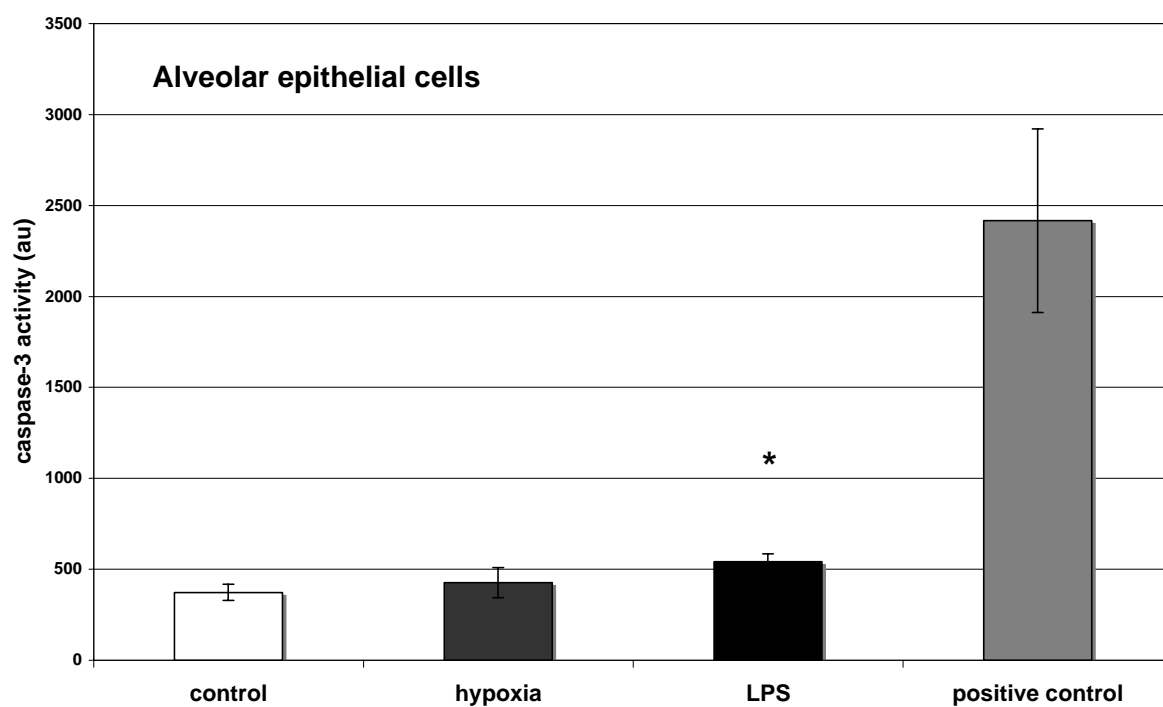


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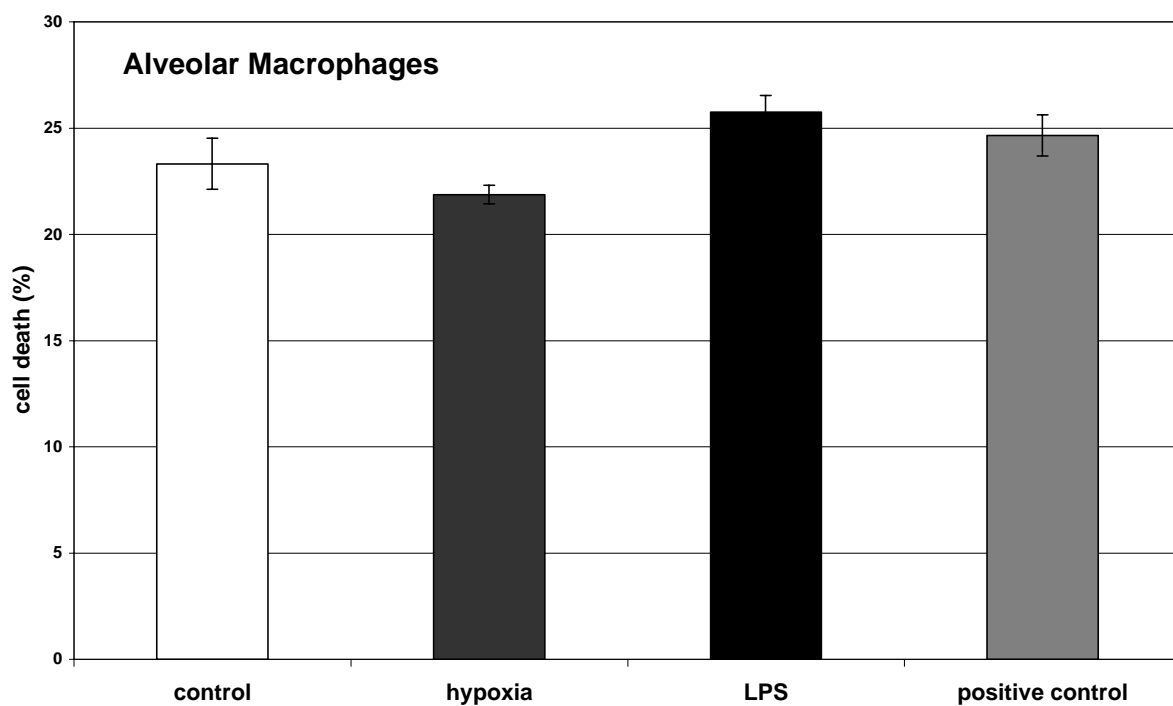


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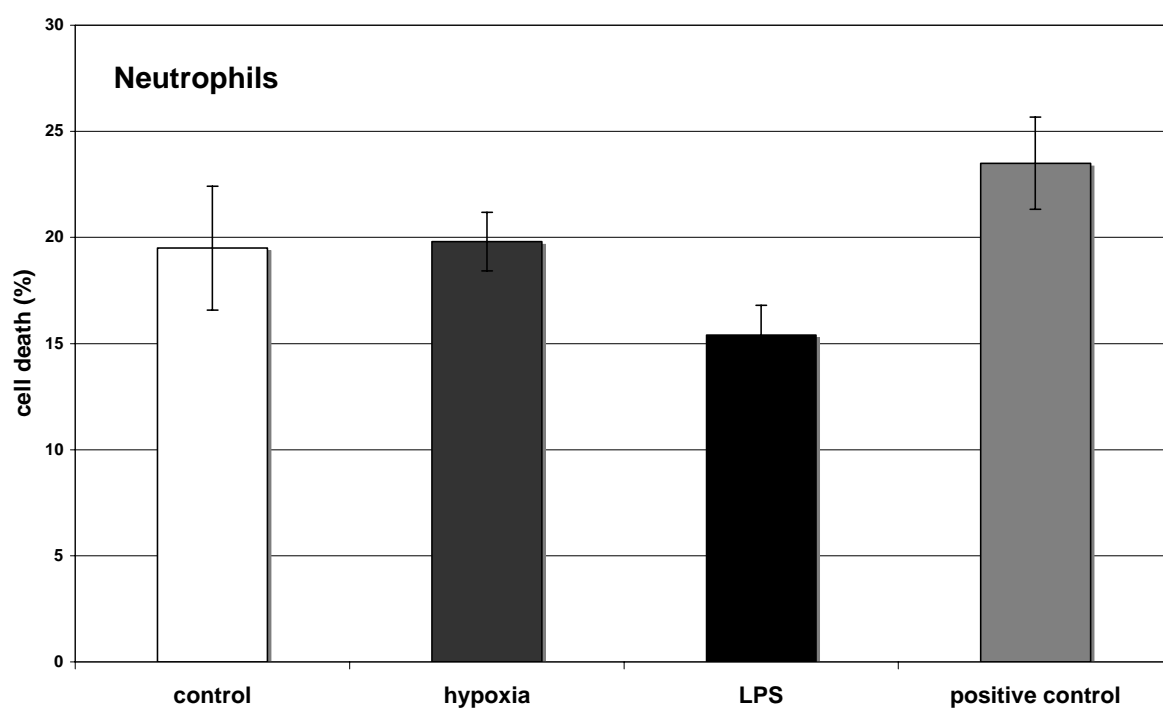


Figure 7

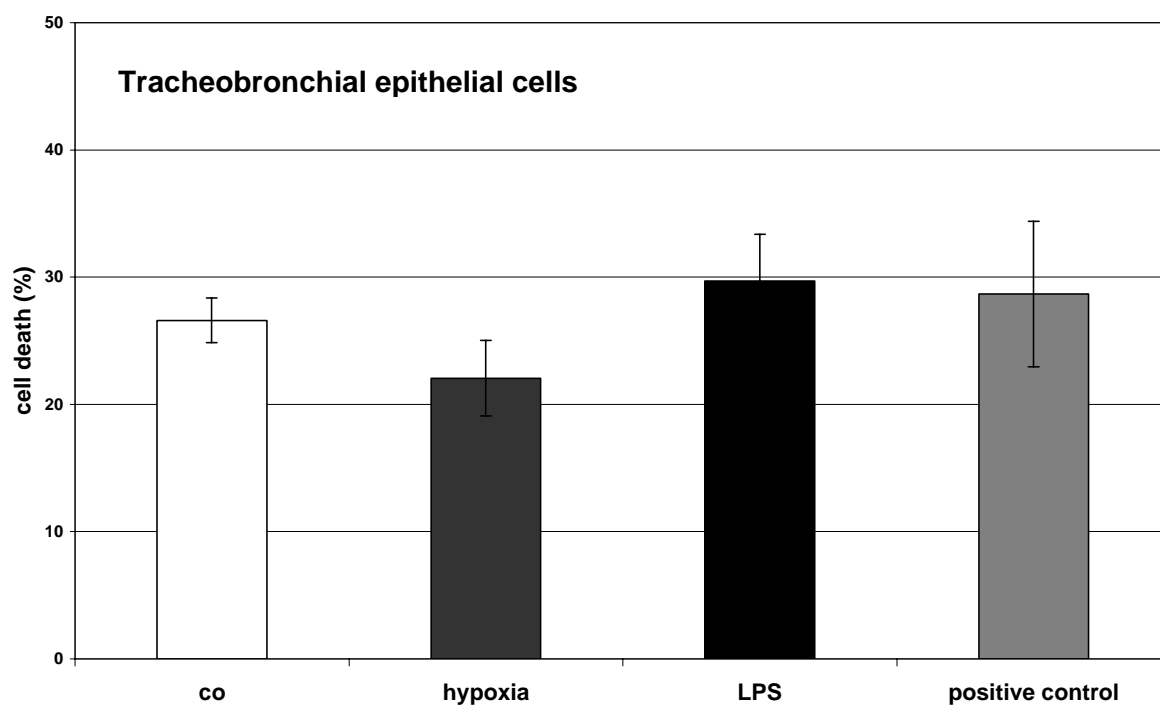
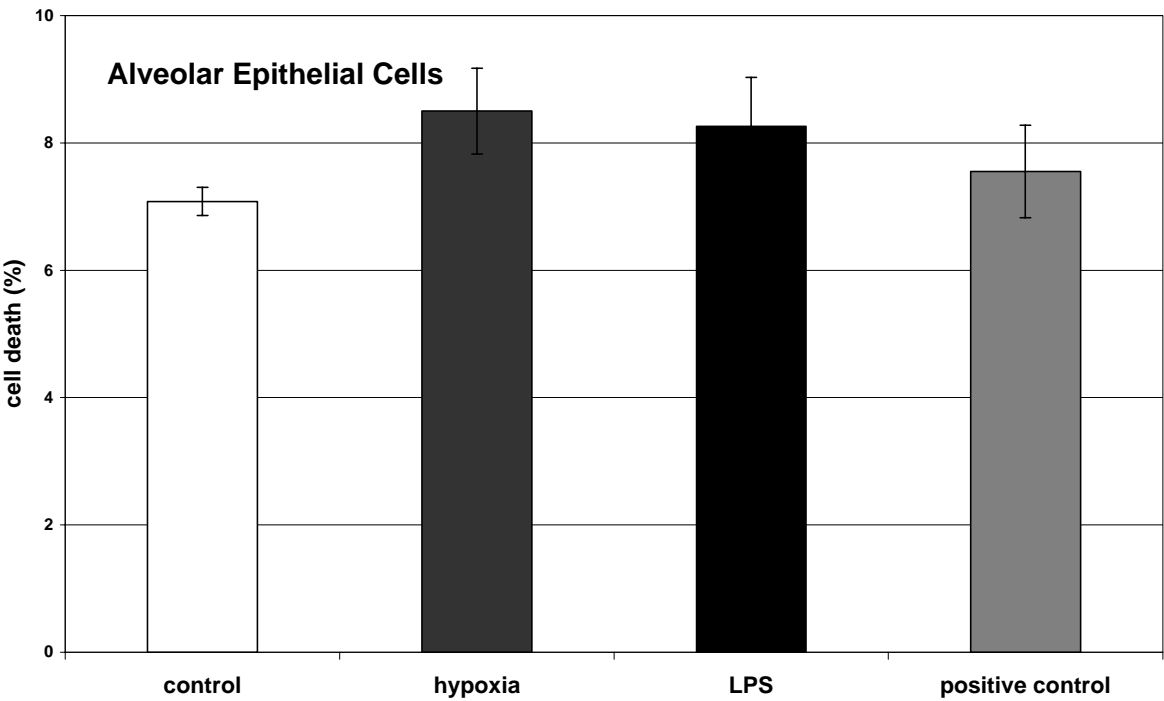


Figure 8



8 Acknowledgement

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9 Curriculum Vitae

Jure Tornic from Zurich ZH

09.02.1981	Born in Zurich
1988-1994	Elementary school in Zurich
1994-2001	Attending the „Kantonsschule Rämibühl“ in Zurich (Matura Typ B)
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